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INTRODUCTION.....

An important goal of current breast cancer research is to develop an in vitro system that can define the mechanisms involved in the progression of human mammary epithelial cells (HMEC) towards a transformed phenotype. In this project, we have focused on an aspect of HMEC behavior that is likely to be involved in this progression, namely, the correct spatial sorting of growth factors and their receptors to discrete cellular locations. We chose this research focus for two important reasons: 1) recent evidence indicates that defects in receptor/ligand trafficking is a hallmark of proliferative disorders in epithelial cells (1,2), and 2) since receptor trafficking is primarily a negative regulatory process defects in this pathway are likely to amplify receptor signaling (3,4). Because correct receptor trafficking depends on the function of many intracellular regulatory systems, it provides a sensitive readout of their status. The EGF receptor system is used as the primary experimental model because it plays a central role in the growth, motility and proliferation of normal HMEC as well as many breast cancers (5-8). Therefore any significant alterations in growth factor regulation in HMEC is likely to perturb the EGF receptor system.

The functions of growth factors extend far beyond simple growth regulation. They are involved in cell differentiation, chemotaxis, morphogenesis, wound healing and gastric acid secretion (9). Originally, growth factors were thought to be products secreted by cells, but in fact, many are produced as membrane-associated precursors. For example, EGF is initially produced as a 170 kDa membrane protein (10) and transforming growth factor alpha (TGF-α) is produced as a 20-22 kDa MW precursor (11). In the case of TGF-α, release from the cell surface occurs through regulated proteolysis (12). The multiple levels at which availability of growth factors can be regulated provide many opportunities for fine control of tissue functions.

Three main routes of growth factor signaling are currently recognized: autocrine, paracrine and juxtacrine (13). In autocrine signaling, cells make both the growth factor and the complementary receptors. In general, the factors must be transported to the cell surface to be functional. In paracrine signaling, different cells make the ligand and receptors. The factor must be transported from the site of production to the site of binding, usually by diffusion. Finally, juxtacrine signaling occurs when receptors on one cell bind directly to the membrane-associated ligand on another cell. All of these types of signaling can be regulated by controlled synthesis, rate of ligand release, and by competition for ligand capture either between different cells or by extracellular matrix proteins (13). Growth factor signaling is also regulated by the physical separation of the ligand and receptor at the cell surface or within the endocytic pathway. This spatial regulation is mediated by sorting components which bind to receptor cytoplasmic domains (14). Growth factors may also be synthesized initially as transmembrane proteins, presumably allowing cells to physically segregate them from receptors.

Epithelial cells display a high degree of spatial organization as evidenced by their polarized phenotype. Kidney, breast and intestinal epithelial cells all show similar features; all are associated through tight junctions and have distinct basolateral and apical surfaces (15). In-vivo, breast epithelium is organized into ducts, ductules and alveoli consisting of a basement membrane, a discontinuous layer of myoepithelial cells, a layer of basal epithelial cells and a layer of luminal cells (16). Both basal and luminal cells display a polarized distribution of integrins and EGF-R (17-19). Integrins mediate interactions with the basement

membrane and appear essential for controlling specific gene expression and maintaining polarization and differentiated functions (20,21). EGF-R are important in regulating epithelial cell growth, and in the breast, are expressed at high levels in myoepithelial cells, basal cells and at the basolateral surface of luminal epithelial cells (19). The functional significance of the basolateral distribution of these receptors is not understood, but could be involved in maintaining the correct organization of epithelial cells within tissues.

Three ligands are thought to be produced in mature breast alveoli which can bind to the EGF-R: EGF, TGF-α and amphiregulin (5,22,23). The best studied of these, TGF-α, is produced by epithelial cells and at least in the mouse, is localized at their basolateral surface (24). Because the basolateral surface contains the EGF-R, the space between this surface and the basement membrane comprises the "microenvironment" in which signaling through the EGF-R occurs. Although EGF is found at high concentrations in the ductal lumen of both mouse and human, little TGF-α is found in breast milk or nipple aspirates of humans (approximately 0.8 ng/ml and 5 ng/ml respectively), indicating a polarized secretion of EGF to the apical surface and TGF-α to the basolateral surface of luminal cells (25). Nothing is known regarding the distribution of amphiregulin in HMEC, but in intestinal cells it displays a luminal distribution (26). Significantly, an extremely high concentration of EGF is found in breast fluids of non-lactating (>200ng/ml) or lactating (100-140 ng/ml) women (25). These concentrations are 2 orders of magnitude higher than the K_d of the EGF-R in HMEC. Therefore, the polarized organization of HMEC segregates their EGF-R from a large reservoir of active hormone.

Ligand activation of EGF-R leads to heterodimer formation between EGF-R and erbB-2, which is thought to result in activation of erbB-2 by transphosphorylation (27-28). Transactivation of erbB-2 also occurs upon the addition of heregulin, a ligand for erbB-3 and erbB-4 (29-32). As observed for EGF, heregulin induces the formation of heterodimers between erbB-3 or erbB-4 with erbB-2, resulting in erbB-2 becoming tyrosine phosphorylated (30, 31 33, 34, 35). There is also evidence to suggest that EGF-R interacts with erbB-3 and erbB-4 (36). Thus, activation of any member of the EGF-R family results in signaling through multiple receptor types. Understanding how these receptors interact with each other is therefore essential to knowing how they work.

The pattern of tyrosine phosphorylation of EGF-R and erbB-2 is important in signal transduction. Specific phosphorylated tyrosines residues serve as docking sites for proteins containing SH2 domains, such as the PI3 kinase p85 subunit and GRB2 (33). The assembly of signaling complexes dictates the subsequent pattern of signal transduction. Intracellular trafficking of the activated EGF-R also regulates receptor activity by controlling the availability of substrates and signaling partners (37). Although ligand binding induces rapid internalization and subsequent lysosomal targeting of EGF-R, it is uncertain whether erbB-2 trafficking is influenced by the EGF-R. It has been suggested that all members of the erbB family are "internalization defective" except for the EGF-R (38), but those studies were done by direct activation of erbB family members or by using chimeric receptors. It has been reported that EGF treatment can stimulate the degradation of erbB-2 in some epithelial cells, but the mechanism of this transmodulation is unclear (39). If EGF-R activation does alter the trafficking of erbB-2, this could be an important mechanism for regulating both the activity and distribution of these signaling molecules in cells. However, the degree of spatial overlap between the members of the EGFR family in mammary epithelial cells is unknown.

The correct distribution of the EGFR within a cell is also very important in regulating signal transduction. Receptors that are mutated to remove sequences that specify receptor internalization can be transforming (4). This is presumably due to an inability to down regulate and thus attenuate signaling from the mutant receptor. However, these mutant receptors may also have access to inappropriate substrates which may contribute to the transformed phenotype.

A great many studies have investigated the relationship between EGF-R and breast cancer (8,19, 40,41). In general, overexpression of the EGF-R in breast tumors indicates poor prognosis, but other growth factor receptors, such as HER2/*neu*, also appear to be linked to breast cancer (8). The incidence of overexpression of the EGF-R is more common than overexpression of HER2/*neu* (45% versus 20% respectively; (8)). Significantly, less than 20% of the tumors that display overexpression of the EGF-R also show amplification of the EGF-R gene, whereas all incidents of HER2/*neu* overexpression appear to be due to gene amplification (42). This indicates that the EGF-R is subject to multiple levels of control that can be independently altered during transformation. Tight control on the EGF-R system is probably necessary because it appears to be the major regulator of HMEC proliferation in vivo. EGF-containing pellets can stimulate normal ductal growth in regressed mammary glands of ovariectomized mice (24). Estrogens appear to regulate the proliferation of HMEC in vivo and in vitro in part through an EGF-R autocrine loop (43,44). Blocking EGF-R occupancy in vitro using a monoclonal antibody causes HMEC to reversibly enter G_0(6). EGF is essential for the motility and assembly of HMEC into organized alveolar structures in vitro. EGF also has a dual effect of promoting growth and chemotaxis/motility of keratinocytes (45) and intestinal epithelial cells (46), suggesting that it has a general role in both establishing and maintaining the structure of epithelial tissues. Recently, rearrangements of the EGF-R have been found in 78% of breast carcinomas (47), again implicating the activity of this receptor in transformation of breast epithelial cells.

Because of the importance of the EGF-R in HMEC regulation, it appears likely that genetic alterations that give HMEC a growth advantage will operate either directly or indirectly through this receptor system. Despite the numerous studies on EGF-R and breast cancer, this idea has not been critically tested. Studies that document the presence or absence of EGF-R (or their overexpression) are not particularly informative in this regard. For example, the MCF-7 breast cancer cell line displays very low levels of EGF-R expression compared to normal HMEC, but estrogen can induce proliferation in these cells in part through an EGF-R/TGF-α autocrine pathway (48). In rapidly proliferating HMEC, there is a positive relationship between TGF-α levels and proliferation, apparently due to a positive feedback loop operating through the EGF-R (5). Amphiregulin and EGF-R levels are also high in proliferating HMEC, but not in intact organoid structures (5,23). Control of receptor number could regulate other aspects of HMEC function, such as directional sensing of ligands. In addition, genetic lesions that operate downstream of the EGF-R itself would not necessarily affect receptor expression. The present uncertainty regarding the role, if any, of the EGF-R in breast cancer reflects our general lack of understanding of its role in normal epithelial cell function, an issue directly addressed by our studies.

Over the last year, we have make progress in our efforts to understand the role of the EGFR distribution in both tissue organization and cancer. We have caused mislocalization of EGFR in polarized epithelial cells and found that regulation and down regulation of the EGFR are distinct from apical and basolateral surfaces. Furthermore, substrate phosphorylation is

distinct from the two cell surfaces. We have also extended our studies on the transmodulation of the erbB-2 gene product by the EGFR. We found that overexpression inhibits down regulation of erbB-2 triggered by EGFR activation. In addition, overexpression of erbB-2 also inhibits normal downregulation of teh EGFR, apparently by inhibiting lysosomal targeting. Finally, we have improved our methodologies for expressing mutant forms of EGFR and its ligands in nontransformed HMEC, providing a basis for further studies on the role of EGFR trafficking in HMEC physiology.

BODY

The tasks in the statement of work are:

> Task 1: Determine the normal pattern of compartmentation and regulation of EGFR and its ligands in nontransformed mammary epithelial cells. Define the extent to which this is similar to the pattern described for other cell types and define conditions under which these cells form organized alveolar structures. (Months 1-24)
>
> Task2: Determine whether oncogenic forms of the EGF receptor found in breast cancer display the same pattern of spatial regulation and biological activity as activated, wild type EGF receptors (Months 12-36)
>
> Task3: Express genetically altered EGF receptors and ligands in mammary epithelial cells (Months 12-36)
>
> Task 4: Demonstrate that mis-sorting or inappropriate expression of the EGFR or its ligands provides a growth advantage to HMEC or inhibit normal organization (Months 24-48)
>
> Task 5: Determine how the pattern of spatial regulation of the EGFR affects its ability to transactivate and transmodulate erbB-2 (Months 12-36)

The last revised annual report was submitted March 27th of this year. That report covered progress on the project up to that date. This report covers the 7 months of work done on the project since the previous report.

TASK 1: Determine the normal pattern of compartmentation and regulation of EGFR and its ligands in nontransformed mammary epithelial cells. Define the extent to which this is similar to the pattern described for other cell types and define conditions under which these cells form organized alveolar structures.

This task has been completed. The previous two annual reports detail the behavior and organization of HMEC. To determine how these results compare with other cell types, we conducted a study of the effect of high receptor expression on another epithelial cell type; LLCPK1 cells. Because this cell type forms tight junctions on transwell filters, it is possible to determine the effect of receptor overexpression on apical/basolateral signaling. This was one of the original aims of this project, but technical limitations of the HMEC system prevented us from using those cell types in this aspect of the project. We therefore completed the proposed experiments using the LLCPK1 cells to determine whether our overall hypothesis was valid.

That is, whether alterations in receptor distribution would result in alterations in receptor signaling.

It is well appreciated that polarized epithelial cells display distinct sets of membrane proteins on their apical versus basolateral surfaces. This distribution is due to differential sorting of newly synthesized as well as recycling proteins. Signals for apical or basolateral targeting reside within the proteins themselves and are recognized by as yet poorly defined cellular mechanisms that operate at several different steps in the membrane trafficking process. It is clear that the correct localization of a number of membrane transporters is essential for the function of most polarized epithelium (15). Abnormal localization of ion transporters is known to be associated with a number of disorders, such as polycystic kidney disease (PKD) (49) and the loss of normal membrane polarity is frequently found in cancer and as a consequence of oncogene expression (15, 50, 51). Far less understood, however, is the role that loss of cell polarity plays in disease itself. Is mislocalization of membrane proteins a symptom or a causal element?

Receptors for growth factors and cytokines, such as EGF also display a polarized distribution in epithelial cells as do the autocrine ligands which activate these receptors (52-54). An assumption underlying models relating abnormal receptor distribution with diseases is that there is a functional consequence of specific receptor mislocalization. Although it is clearly necessary to have receptors colocalized with their activating ligand, the ability of receptors to signal could also depend on their coincidence with substrates (55). In addition, the ability of regulatory molecules to modulate receptor signaling could also depend on their colocalization. To date, however, no studies have addressed the consequences of receptor mislocalization on their regulation and signaling.

Materials and Methods

Cell lines - the pcCMV3/EGFR plasmid was obtained from Dr. Gordon Gill. It was transfected into the LLCPK1 cell line by calcium phosphate. Resistant colonies were isolated by growth in medium containing 1.6 mg/ml G418. colonies were picked and grown in α-MEM (ICN) with 10% fetal calf serum (Hyclone) and screened by flow cytometry.

To measure receptor number, cells were plated at 50,000 cells per well onto polycarbonate filters (0.45um Transwell; Costar) in α-MEM (ICN) with 10% fetal calf serum (Hyclone), penicillin, streptomycin, and glutamine. At 14 days in culture, the medium was changed to cold α-MEM-HB (1gm/L bovine serum albumin and 20mM HEPES buffer) for 30 minutes and the transwell resistance was tested to ensure an intact monolayer. Only wells measuring 350 megaohms or above were used. Cells were incubated to equilibrium at 0°C with saturating concentrations of ^{125}I-EGF (12 nM) applied to either the apical or basolateral surfaces labeled to approximately 150,000 cpm/ng as described (57). Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled EGF.

Mitogenic response of polarized K2 cells - Polarized monolayers were changed to medium containing 0.5% serum overnight. Following addition of 15 nM EGF to either the apical or basolateral transwell chamber, 2μCi/ml of [^{3}H] thymidine was added at 18 h to both apical and basolateral chambers. After 2 h, 10% trichloroacetic acid was added and acid insoluble cpm were measured. In some cases, the anti-EGFR antagonistic monoclonal antibody 528 (10 μg/ml) was added at 0 time to the indicated chamber.

Internalization and down regulation of EGFR - Polarized monolayers of K2 cells on transwell

inserts were incubated with 10 ng/ml of ^{125}I-EGF (150,000 cpm/ng) added to either the apical or basolateral chamber for up to 5 min at 37°C. At 1 min intervals, the inserts were rinsed in ice-cold phosphate-buffered saline 6 times and the membranes removed using a #4 cork bore. Surface radioactivity was removed by placing the membranes in 1.5 ml acid strip solution (50 mM glycine/HCl (pH 2.5), 150 mM NaCl, 2 M urea) for 8 minutes. Internalized radioactivity was solubilized using 2.5 ml 1N NaOH. The relative amount of internalized and surface associated EGF was converted to internalization plots as previously described (56). Nonspecific EGF binding was determined in parallel using a 1000-fold excess of unlabeled EGF. Down-regulation of surface EGFR was determined by taking polarized monolayers of K2 cells and incubating them with 100 ng/ml unlabeled EGF added to either the apical or basolateral chamber at 37°C for different periods of time. Cells were rinsed twice with phosphate-buffered saline and then incubated a further 2 hr on ice with 100 ng/ml of ^{125}I-EGF added to the same chamber as the unlabeled EGF. Membranes were then rinsed 6 times with ice-cold phosphate buffered saline, removed with a #4 cork bore and solubilized in 2% SDS prior to counting.

Total phosphotyrosine of K2 cells treated with EGF - EGF (15 nM) in complete medium was added to either the apical or the basal chamber of 3 Transwells for 15 minutes. The cells were lysed in ice-cold lysis buffer (50mM HEPES, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 100mM NaF, 10mM Na_2PO_4, 1mM Na_3VO_4, 10% glycerol, 1%Triton X-100, aprotinin, leupeptin, chymostatin, pepstatin) α-MEM, sonicated for 10 seconds, then clarified at 10,000 rpm at 4°C for 15 minutes. 60ug (total protein) of each lysate (and control lysates from untreated cells) was separated on 7.5% polyacrylamide SDS gel and electrophoresed (57). After transfer to nitrocellulose, phosphotyrosine was detected using antibody PY20 (Transduction), followed by a rabbit-anti-mouse monoclonal antibody (Zymed), and detected with ^{125}I-protein A. The blot was scanned on a Phosphorimager (Biorad).

SHC phosphorylation - Polarized monolayers were treated with 15 nM EGF for 10 min at 37°C, rinsed and groups of 3 wells were extracted with a total of 1 ml of ice-cold lysis buffer, and clarified by centrifugation and incubation with sepharose coupled to an irrelevant antibody. After equalizing the protein concentrations, SHC was immunoprecipitated by 2 μg anti-SHC antibody (Transduction laboratories) and 20μl of protein A sepharose using a 1.25 h incubation.

Phosphorylation of FAK and beta-catenin - Polarized monolayers of K2 cells were treated with or without 100ng/ml of EGF added to either the apical or basolateral chambers for 15 minutes. Triplicate groups of cells were lysed in ice-cold lysis buffer, sonicated for 10 seconds, then clarified at 10,000 rpm at 4°C for 15 minutes. FAK antibodies or beta-catenin mAb were added to the lysates and incubated on a rocker at 4°C overnight (total of 4μg/sample; Transduction laboratories). Then, 50ul of a 50% slurry of Protein A/G- Sepharose beads (Immunopure; Pierce) was added to each lysate and incubated on a rocker at 4°C for 2 hours. The beads were washed three times in 1ml of lysis buffer then boiled in SDS-reducing sample buffer, resolved on SDS-7% acrylamide gels, transferred to nitrocellulose, then probed with either antiphosphotyrosine mAb , or beta-catenin or FAK mAb. The blots were quantified using a Molecular Imager storage phosphor device (Bio-Rad Laboratories).

Results and Discussion

We found that overexpression of EGFR in LLCPK1 cells caused the missorting of EGF receptors to the apical surface. The parental LLCPK1 cell line express approximately 30,000

EGFR per cell (~22,000 basolateral and ~5,000 apical EGFR per cell) as determined by ^{125}I-EGF binding studies (Fig. 1A). Transfection of LLCPK1 cells with a vector containing the full-length human EGFR resulted in stable transfectants (K2 cells) expressing approximately 1.5×10^6 EGFR per cell basolaterally and 8×10^5 apically, as determined by ligand binding analysis at 0°C (Fig. 1A). Thus, K2 cells expressed many more EGFR apically than the total receptor complement of the parental cell line.

We next determined whether the apical and basolateral EGFR could be activated to similar degrees. Cells on permeable filter inserts were serum-starved for 18 hours after which EGF was added either apically or basally for 15 minutes. Tyrosine phosphorylated proteins, which include the EGFR, were detected by western blot for K2 cells stimulated with EGF added apically or basally (Fig. 1B). Some basal tyrosine phosphorylation of the EGFR was also seen in the absence of exogenous EGF, probably reflecting autocrine growth factor production and release. Although no readily apparent differences in phosphotyrosine patterns were observed between apically and basally stimulated cells, the total extent of tyrosine phosphorylation paralleled the 2:1 ratio of basolateral versus apical EGFR levels. This indicates that the kinase activity of the EGFR on a per receptor basis is similar at both the apical and basolateral cell surface.

To determine whether apical and basolateral EGFR were both capable of generating a biological response, we examined the mitogenesis of cells following either apical or basal EGF stimulation. As shown in Fig. 2A, similar levels of ^{3}H-thymidine incorporation were observed in response to either apical or basolateral EGFR activation. The simultaneous addition of EGF and an antagonistic anti-EGFR antibody to the ipsilateral side of the monolayer resulted in a significantly diminished cellular response. Addition of anti-EGFR antibody to the contralateral side had no effect. This demonstrated that there was no significant leakage of EGF through the polarized epithelium.

In contrast to the similar mitogenic response to either apical or basolateral EGF addition, phosphorylation of immunoprecipitated SHC was more extensive when EGF was added to the basolateral side (Fig. 2B). EGFR which co-precipitated with SHC also showed more extensive tyrosine phosphorylation when EGF was added to the basolateral size. Both ERK1 and ERK2 were tyrosine phosphorylated to the same degree in response to apically or basally added EGF as assessed by immunoprecipitation and western blot analysis (data not shown). Because of the high constitutive level of activation of these substrates, however, it is difficult to determine whether their activation is proportional to the level of receptor occupancy. These data do suggest that overexpression of the EGFR in LLCPK1 cells is not toxic and does not lead to abnormally high levels of substrate phosphorylation.

We then looked at cell responses to EGFR stimulation other than proliferation that might be spatially restricted. Because trafficking of membrane proteins is known to be different from the apical and basolateral surfaces of polarized cells, we first examined endocytosis and downregulation of the EGFR. As shown in Figure 3A, endocytosis of the EGFR was significantly faster at the basolateral than apical cell surface. To ensure that the faster rate at the basolateral surface reflected a differential distribution of EGFR-specific regulatory molecules and not simply a different net rate of endocytosis from the two surfaces, we expressed the c'973 EGFR in LLCPK1 cells. This receptor mutant has intrinsic tyrosine kinase activity, but lacks the domains necessary for specific ligand-induced endocytosis (3, 4). As shown in Figure 3B, the c'973 EGFR displayed no difference in apical versus basolateral

endocytosis. These data suggest that EGFR-specific internalization components are primarily restricted to the basolateral surface of cells.

If apical EGFR do not have access to proteins that mediate ligand-induced endocytosis, then one would predict that they would not be able to undergo efficient receptor downregulation. To test this, we incubated cells with high concentrations of EGF and then looked at the number of receptors remaining on the cell surface as a function of time by ^{125}I-EGF binding at 0°C. As shown in Figure 3C, basolateral EGFR showed a continuous decrease in numbers for the entire incubation period, reaching approximately 20% of initial numbers. In contrast, the apical EGFR displayed an initial drop, most likely due to receptor occupancy, but then remained constant at 60% of the initial receptor levels. These data suggest that EGFR downregulation is indeed less efficient from the apical than from the basolateral surface.

Cell adhesion structures such as focal adhesions and adherens junctions are localized to the basal and lateral cell surfaces, respectively, and contain known EGFR substrates. Focal adhesions are localized to the basal cell surface where they provide connections between the extracellular matrix and cytoskeleton, and contain focal adhesion kinase (FAK) which can be tyrosine phosphorylated in response to *src*, integrin binding, and neuropeptides (58). Adherens junctions are formed along the lateral cell surfaces at cell-cell junctions, basal to the tight junctions, and are associated with the EGFR substrate beta-catenin (59). To determine whether these structurally-associated proteins might interact differently with apical versus basolateral EGFR, we examined the phosphorylation of FAK and beta-catenin. After addition of EGF to either the apical or basal surfaces, we extracted the cells and measured the phosphotyrosine levels of immunoprecipitated FAK and beta-catenin by western blots. As shown in Fig. 4A, FAK displayed measurable phosphotyrosine levels even in the absence of exogenous EGF, similar to the case with SHC (see Fig. 2B). Although the addition of EGF to the apical surface had little effect on FAK tyrosine phosphorylation, the addition of EGF to the basolateral surface caused a significant increase in FAK phosphotyrosine content. In contrast, beta-catenin was tyrosine phosphorylated to a much greater degree in response to apical rather than basal EGF addition (Fig. 4B). These results demonstrate that access to specific tyrosine kinase substrates is different at the apical versus basolateral surface of polarized cells.

Polarity enables a number of important functions of epithelial cells, such as domain specific (apical versus basolateral) calcium signaling (60) and vectorial transport of solutes (61). Our results demonstrate that another feature of polarized epithelial cells is a restriction of EGFR-phosphorylated substrates. Beta-catenin and FAK are both important cell signaling proteins, but their distribution within cells is dictated in part by interacting with spatially restricted cellular structures. It is perhaps not surprising that access of the EGFR to these substrates would differ between apical and basolateral surfaces. If the normally low numbers of apical EGFR are activated in polarized intestinal epithelial cells *in vitro* , mitogenesis or tyrosine phosphorylation of EGFR substrates was not observed (Damstrup et al., unpublished results) Therefore, beta-catenin is probably not a normal substrate of apical EGFR. However, it could become an EGFR substrate if cell polarity or tight junctions were disturbed, allowing EGFR to be distributed over the entire cell surface. Along these lines, overexpression of *v-src* resulted in excessive tyrosine phosphorylation of beta-catenin and decreased cell-cell interactions (62).

In addition to the EGFR, beta-catenin interacts with alpha-catenin, E-cadherin, and the tumor suppressor APC gene product in mammalian cells, and, is involved in the

wnt/wingless signaling pathway (63, 64). Beta-catenin has been localized to the lateral cell membrane in polarized epithelial cells, but cytoplasmic concentrations of the protein appear to be regulated by the APC tumor suppressor protein (65). In K2 cells, beta-catenin is localized to the lateral cell membrane as evaluated by XZ-plane confocal microscopy, but cytoplasmic staining was seen as well (data not shown). The fact that apical EGFR stimulation resulted in a rapid and large increase in tyrosine phosphorylation of these proteins suggests that the apical EGFR has access to a cytoplasmic pool of beta-catenin. Because overexpression of EGFR causes its mislocalization, this may result in a consequent modification of normally inaccessible substrates such as beta-catenin.

In addition to differences in substrate phosphorylation patterns, we also found that the internalization of EGF and EGFR downregulation were distinct between the apical and basolateral surfaces of cells. The most likely explanation for the differences in the internalization rates of the EGFR at the apical and basolateral surfaces is a differential distribution of the internalization components. The low rate of endocytosis of the EGFR from the apical surface is probably responsible for the observed low extent of apical receptor downregulation.

A lower extent of receptor downregulation at the apical surface of K2 cells would imply a higher level of receptor activity at steady state. This is consistent with our observation that the mitogenic response of K2 cells to EGF is equivalent at the apical and basolateral surfaces despite a 2-fold greater number of basolateral EGFR. Therefore, overexpression or mislocalization of EGFR in polarized epithelium would not only allow access to inappropriate substrates, but would also reduce attenuation of receptor signaling. Both processes could give rise to inappropriate cellular responses. In addition, apical EGFR would have access to the normally high levels of luminal EGFR ligands (66, 67). Together, these factors could explain why EGFR overexpression is associated with epithelial tumors and poor prognosis (8). Therefore, rather than only being an effect of aberrant cell regulation, EGFR mislocalization may play a causal role.

Task2: Determine whether oncogenic forms of the EGF receptor found in breast cancer display the same pattern of spatial regulation and biological activity as activated, wild type EGF receptors. In the last period of the grant, we examined the ability of an oncogenic form of the EGF receptor (Δ2-7 EGFR), which lacks a portion of the extracellular binding domain encompassing exons 2-7, to modify the behavior of normal HMEC. These constitutively activated receptors did not undergo the accelerated internalization and lysosomal targeting displayed by activated wild type receptors. Thus our expectation was that these receptors would show a greater biological activity than the normal EGF receptors. We found that contrary to expectations, the Δ2-7 EGFR significantly inhibited the growth of nontransformed HMEC. We initially concluded that these oncogenic receptors are "defective" receptors and transmit a qualitatively different signal than their normal counterpart. The altered signal is recognized by normal cells, leading to apoptosis and growth inhibition. Transformed cells do not see the signal generated by the Δ2-7 as defective (due to a defect in the signal "proofreading" system in the cells). This leads to altered growth and behavior of the cells expressing the Δ2-7 receptors. We are currently preparing these observations for publication.

One important control that was not accomplished last year was to evaluate the effect of overexpression of wild-type EGFR in HMEC. Although unlikely, it appeared possible that overexpression and activation of normal EGFR would have the same effect as expression of the

Δ2-7 EGFR. In this scenario, the presence of high levels of activated receptors was responsible for the slower growth rate and behavioral alterations in HMEC, rather than the presence of defective receptors. We therefore created HMEC cell lines that expressed high levels of wild type EGFR.

Materials and Methods

HMEC 184A1L5 cells grown in medium DFCI-1 were transduced with retrovirus containing the WT EGF-R and G418 resistance (a kind gift of H.-J. Su Huang of the Ludwig Cancer Institute). They were selected in medium containing 100 μg/ml G418, but lacking EGF. Once clones were isolated, they were examined by flow cytometry for the level of Δ2-7 at the cell surface using the EGFR specific monoclonal antibody 225 followed by FITC-labeled secondary antibody (68). Scatchard analysis of cell lines was performed as previously described (69).

Growth curves were generated by counting cell samples every 24 hour using a Coulter counter. Prior to starting the measurements, cells were maintained for 48 h in DFCI-1 medium lacking EGF (70). At time zero a cell sample was taken and either 10 μg/ml monoclonal antibody 225 or 12.5 ng/ml EGF was added to duplicate plates of cells. Cell counts were taken over a 6 day period.

Results and Discussion.

Cells were transduced with the retrovirus containing the WT EGFR gene and selected in medium containing G418. After about a week in selective medium, individual clones were isolated by limiting dilution. Clones were then evaluated by flow cytometry and Scatchard analysis. The line expressing the highest levels of EGFR (line AXR1) was then used for further analysis.

As shown in Fig. 5, the parental A1-1 cell line expressed approximately 250,000 EGFR per cell. The AXR1 line expressed nearly 10-fold greater receptor number at 2,500,000. Both lines displayed biphasic Scatchard plots, indicating multiple affinity classes of receptors (69). Interestingly, the relative fraction of high affinity and low affinity receptors was the same in the two lines, indicating that the high affinity receptor state is not due to saturation of a limiting cellular protein. The overexpression of WT EGFR was confirmed by both flow cytometry and western blot analysis (data not shown).

The growth rates of the AXR1 and parental A1-1 cells were then compared. Cells were incubated in the presence of either no EGF, 1 μg/ml EGF or 10 μg/ml of the antagonistic anti-EGFR antibody 225. As shown in Fig. 6, the A1-1 and AXR1 cells grew at very similar rates in the absence of EGF. Likewise, the addition of EGF stimulated the growth of both cell lines, although the effect was more pronounced in the case of the A1-1 cells. The addition of the antagonistic anti-EGFR mAb 225 also inhibited the growth of both cell lines, showing that activation of the EGFR are required for both the parental and overexpressing cell lines.

Several conclusions can be made from this data. The first is that activation of high numbers of EGFR in HMEC is not growth inhibitory. Although the extent of growth stimulation was not as pronounced as in the parental cell line, EGF addition clearly did not inhibit the growth of the HMEC. Thus the pronounced inhibition of cell growth we observed in cells expressing the constitutively active Δ2-7 EGFR was not simply due to high levels of activated EGFR. This supports our hypothesis that the Δ2-7 EGFR is a "defective" receptor and

generates a qualitatively different signal than a normally activated EGFR.

The second conclusion that can be made is that overexpressing the EGFR does not allow cells to grow in the absence of ligand. Blocking receptor activation by using the antagonistic 225 mAb inhibits growth of receptor overexpressing cells as effectively as the parental cells. It has been known that EGFR overexpression is associated with poor prognosis in breast cancer. It has been assumed that this reflects a degree of growth factor independence that results from receptor overexpression. This is clearly not the case. The AXR1 cells retain their dependence on EGFR ligands. An alternate hypothesis is that overexpression of EGFR results in inappropriate receptor attenuation or regulation. Studies are currently underway to determine whether this is the case with the AXR1 cells.

TASK 3: Express genetically altered EGF receptors and ligands in mammary epithelial cells

Work in prior years has shown that the only workable method for expressing genes in HMEC is to use retrovirus. Therefore, for the last year we have worked hard to master the methodology that will allow us to generate retrovirus in our own laboratory. Until now, the receptor and ligand-containing retrovirus we have used in our experiments have been obtained from other investigators. Because we need retrovirus encoding a variety of different customized EGFR and EGFR ligand mutants, this approach is no longer possible.

Last year we generated a FLAG epitope cassette to allow us to discriminate between mutant receptors (generated by us) and the endogenous EGFR in HMEC. This has been completed as was described in the last report. A second approach that we implemented in parallel with the epitope tagging efforts was to use the chicken EGFR (cEGFR) as a mutagenesis target. First, though, we needed an antibody that would react to the cEGFR, but not the human form. This was successfully accomplished by the synthesis of a peptide to sequences 139 to 154 in the cEGFR. This peptide was then coupled to keyhole limpet hemocyanin and used to generate a polyclonal antiserum. We verified by western blot analysis that this antibody would react to cEGFR, but not to the human form of the EGFR. Efforts are currently underway to affinity purify the antibody for use in immunocytochemistry.

Inserting the cEGFR into a retrovirus - The cEGFR was obtained from Dr. Nita Maihle (Mayo) and placed into Bluescript, generating EGFR/BS. To insert the cEGFR into the MFG retrovirus, the 5′ and 3′ ends of the gene had to be modified to Nco1 and BglII sites respectively. These compatible cohesive ends were generated by PCR cloning. A BglII site was created at the end of the cEGFR gene by amplifying primers containing the new site, cutting the product with Bcl1 and gel purifying the correct fragment. The cEGFR/BS was cut with HindIII, blunt ended, the a T-overhang was added. It was then cut with Bcl1 and gel purified. The cEGFR/BS fragment was then ligated to the purified PCR product. The correct clone was then picked, amplified and verified by sequencing.

The 5′ end of the cEGFR was changed to BspH (compatible with Nco1). The same protocol as above was used, but this time we cut the PCR product with AflIII. The parent plasmid was cut with XbaI, blunt ended, T-overhangs were added and the plasmid was then cut with AflIII. The cut PCR product was then ligated into the parent plasmid. This was cloned, amplified and verified by sequencing.

The MFG retrovirus vector was cut with NcoI and BamH1 to remove the stuffer sequence, and gel purified. The modified cEGFR/BS was cut with BspH and BglII, the appropriate fragment was gel purified and ligated into the MFG vector (71). The cEGFR/MFG

vector was amplified, purified and used to transform the ΨCrip packaging cell line (originally obtained from R. Mulligan) as described (72). Clones of transfectants were isolated and screened for those producing the highest titer. Colonies were isolated from serially diluted plates and grown to confluence in 24 well dishes. The virus-containing supernatants and the cells were frozen until all could be screened at one time.

Screening was done by seeding 184A1 cells into 24 well plates at a density of 10,000/well. When cells were almost confluent, the virus-containing supernatants were rapidly thawed at 37 °C and polybrene was added to a final concentration of 4 μg/ml. Medium from the screening cells was removed, replaced with the test virus stocks, and cells were incubated for 4 h at 37°C with shaking every 30 min. The test virus stocks were then removed and replaced with fresh medium for an additional 2 days. The cells were then screened for the expression of the cEGFR by immunoperoxidase. Cells were fixed with paraformaldehyde, blocked with 1% BSA and incubated with 10μg/ml affinity purified antibody to the cEGFR. Alkaline phosphatase-conjugated secondary antibody was used in conjunction with Chromogen reagent to visualize the positive cells. The 4 producing lines that yielded the highest frequency of positive cells were thawed, expanded and used to produce large quantities of retrovirus for transducing cells.

We are currently using these virus stocks to generate HMEC cell lines that express the cEGFR. Endogenous EGFR will then be inactivated by the addition of antagonistic 225 mAb. Once we have verified that the cEGFR will support the normal biological activities of EGF in the HMEC, then we will mutagenize the cEGFR by PCR-based mutagenesis and determine the effect of the genetically-altered EGFR in mammary epithelial cells. If the cEGFR does not generate a normal biological activity (unlikely), then we will put all of our efforts into the FLAG-labeled human EGFR construct outlined above.

Generation of different mutant of EGFR ligands This work is well underway. Several of these mutants have been inserted into retrovirus and expressed in both luminal and basal HMEC. The basic strategy is to insert an epitope tag (FLAG) at the carboxy terminus of the ligand. This allows us to track the fate of the ligand tail once the core domain of the ligand (which binds the receptor) is released. We have also inserted a Myc epitope tag at the amino terminus of the ligand to allow us to "capture" the ligand using exogenous anti-epitope antibodies. This approach will allow us to evaluate the fate of different ligand domains following processing and to interfere with autocrine signaling in a meaningful way.

One interesting observation made in the previous year was that expression of chimeric mutants caused apparent growth inhibition of HMEC. We are currently investigating this interesting observation.

Task 4: Demonstrate That Mis-sorting or Inappropriate Expression of the EGF Receptor or its Ligands Provides a Growth Advantage to Normally Organized Epithelial Cells

This aspect of the project requires the successful expression of receptor and ligand mutants in cells. We are currently completing the methodological aspects of this task (see task 3 above). Experiments addressing this specific task are the subject of most of the work for the next year.

Task 5: Determine how the pattern of spatial regulation of the EGFR affects its ability to transactivate and transmodulate erbB-2

This task is mostly completed. During the previous period of the project, we investigated the domains of the EGFR which control transmodulation of erbB-2 and have determined how trafficking of the EGFR influences transmodulation of erbB-2. This study has been published (68). During the last year, we have extended these studies to include an investigation of the affect of overexpression of either EGFR or erbB-2 on their ability to transmodulate each other. The reason for this is that we showed that overexpression of either erbB-2 or EGFR causes a primary redistribution of these proteins to the cell surface (68). We were interested in the effect this redistribution would have on transmodulation.

Ligand binding not only activates the EGF receptor but also initiates negative regulatory processes. In this part of the project, we examined negative regulation by receptor degradation resulting from intracellular trafficking to the lysosomes. While activation of erbB-2, erbB-3 and erbB-4 have been well characterized, the negative regulation of these receptors has not been extensively studied. Conflicting reports have been published regarding the trafficking of erbB-2 following transactivation with EGF. We and others have described effective downregulation of erbB-2 by lysosomal targeting in three different cell types (68, 73). In contrast, studies with chimeras composed of the EGF-R extracellular domain and the erbB-2 cytoplasmic domain have shown that EGF has no affect on the internalization or degradation rate of the chimeric receptor (74). Another study measured the downregulation of erbB-2 in SKBR-3 cells that contain a 100 fold erbB-2 gene amplification (75). This study found that although erbB-2 was transactivated following EGF treatment, there was no measurable decrease in erbB-2 half-life (75). Instead of interpreting this result to mean that degradation of erbB-2 is not part of its negative regulation, we alternatively suggest that lysosomal targeting is a mechanism of erbB-2 downregulation, but normal regulation is impaired when erbB-2 is overexpressed.

Materials and Methods

General - Polyclonal rabbit antibody N13 directed against a peptide corresponding to residues 1-13 in human EGF-R was a gift of Dr. Debora Cadena. Polyclonal rabbit antibody 1917 to erbB-2 directed against a peptide corresponding to the 18 carboxy terminal residues of human erbB-2, were provided by Dr. Gordon Gill. Polyclonal rabbit antibodies specific for phosphotyrosine were generated and affinity-purified as described (76).

Cell Culture - B82 mouse L cells transfected with normal (WT) or mutated (M^{721}, c'647, c'958, and M^{721}c'958) human EGF receptors were a generous gift of Dr. Gordon Gill. Their construction was described previously (3). The human mammary epithelial lines MTSV and ce2 were a gift from Joyce Taylor-Papadimitriou and were grown as described (77).

Quantification of EGF-R , erbB-2 and phosphotyrosine levels - Confluent cultures of cells were rinsed and lysed in an NP-40 buffer (150mM NaCl, 1% NP-40, 50mM Tris pH 8) and debris removed by centrifugation. Samples were brought to 2% SDS, 1% β-mercaptoethanol and heated to 100°C for 5 min. Equal amounts of total cellular protein from each sample were separated on 5-7.5% gradient gels and transferred to nitrocellulose. EGF-R and erbB-2 were detected by N-13 and 1917 polyclonal antisera respectively using ^{125}I-labeled protein A as described (68). The concentrations of and incubation times with ^{125}I-labeled protein A were chosen to be in the linear range of the protein load of the gels. The blots were analyzed by storage phosphor plates using the Bio-Rad G250 Molecular Imager. The Bio-Rad Molecular Analyst package was used to quantify the amount of radioactivity associated with each band. In some cases, EGF-R levels were determined by a sandwich ELISA as previously described

(78). In this case, cells were extracted for 10 min at 0°C in 20 mM CHAPS, 10 mM HEPES buffer (pH 7.4), 4 mM iodoacetate and 100μg/ml each of leupeptin, chymostatin, pepstatin and aprotinin. Standard curves were generated using extracts from B82 cells expressing a known level of EGF-R protein and using receptor-negative B82 cells as blanks.

To determine the tyrosine phosphosphate content of erbB-2 protein, cells were lysed for 10 min at 0°C in 1% Triton X-100, 150 mM NaCl, 50 mM Hepes (pH 7.2), 10% glycerol, 1 mM Na_3VO_4, 4 mM iodoacetate and 10μg/ml each of chymostatin, pepstatin, leupeptin and aprotinin. After centrifugation for 10 min at 10,000 x g at 0°C, the 1917 anti-erbB-2 polyclonal antiserum was added (1:100) followed by rocking for 3 h followed by the addition of 100 μl of protein A sepharose (50% slurry) for an additional hour. The beads were washed several times in lysis buffer and boiled in SDS sample buffer prior to gel electrophoresis and transfer to nitrocellulose as detailed above. Phosphotyrosine was detected using affinity-purified polyclonal antibodies and erbB-2 protein was quantified in a parallel blot using 1917 antiserum. The Bio-Rad G250 Molecular Imager and the Molecular Analyst package was used to quantify the amount of radioactivity associated with each band.

Results and Discussion

ErbB-2 overexpression inhibits its downregulation To directly assess if erbB-2 amplification affects its negative regulation, we employed a human mammary epithelial cell line that has been transfected with the erbB-2 gene to cause overexpression. These cells (MTSV) are derived from human breast milk, have been immortalized with SV40 large T antigen, but are not malignantly transformed (77). The parental MTSV and overexpressing cell lines (ce2) express comparable numbers of EGF receptors while erbB-2 expression is increased approximately 6 fold.

Downregulation experiments were conducted by treating both cell types with 100ng/ml EGF for 24 hours to transactivate erbB-2 and induce its downregulation. A well characterized fibroblast cell line (B82; 3) was included for comparison. Downregulation was assessed by western blot for erbB-2 shown in Fig. 7A. Both the fibroblast and the parental MTSV cell line downregulate erbB-2 by 75% in response to 24 hours EGF treatment. However, the erbB-2 overexpressing cell line only downregulates the receptor 30% in 24 hours. To more rigorously examine this observation, we measured erbB-2 downregulation over a shorter time course. Fig. 7B shows that while the parental cell line has downregulated erbB-2 by 75% within 6 hours, the erbB-2 overexpressing cell line has downregulated this receptor only 10%. Thus, erbB-2 amplification prevents effective downregulation of the receptor. Inhibition of erbB-2 downregulation as a result of receptor overexpression is similar to what has been described for the negative regulation of the EGF-R. A431 cells are an squamous cancer cell line which contains an amplified EGF-R. When activated with EGF, the EGF-R's in these cells are not efficiently internalized or degraded in the lysosome (79). The results presented here, combined with previously reports, demonstrate effective erbB-2 downregulation in four non overexpressing cell types: fibroblast, HC11, HMEC184A1L5, and MTSV (68). However, overexpression of erbB-2 abrogates this negative regulatory process as is observed here, in ce2 cells, and previously reported in SKBR-3 cells (75).

It has been proposed that downregulation of the EGF-R is inhibited in A431 cells because there are specific trafficking molecules that are not present in sufficient quantity to mediate downregulation of large numbers of EGF receptors (79). Downregulation of erbB-2 could be reduced by an analogous mechanism in which a molecule required for its trafficking

is limiting. Alternatively, erbB-2 amplification may inactivate the downregulatory pathway. To determine if the erbB-2 degradation pathway was functional in these cells, we reanalyzed data from Fig. 7A. The phosphorimager signal from EGF treated cells was subtracted from that of untreated cells to yield the amount of receptor degraded over a 24 hour time period, and is plotted in Fig. 8. We observed that both cell lines degrade similar amounts of erbB-2, indicating that this negative regulatory process is functional when erbB-2 is amplified. Instead, it is the percentage of erbB-2 degraded that is reduced in the overexpressing cell line. This is consistent with a scenario in which the parental MTSV cells are operating at maximum capacity with regard to erbB-2 degradation. Additional activated erbB-2 are not able to be downregulated, possibly because a trafficking molecule, analogous to that described for the EGF-R, is limiting.

The half-life of erbB-2 was measured to confirm that loss of receptor protein was indeed due to EGF induced lysosomal degradation, as is well characterized for the EGF-R (57) and previously reported for erbB-2 in other cell types (68). The 6 hour half-life of erbB-2 in parental MTSV cells is reduced to 1 hour in the presence of EGF. (Fig. 9) This indicates that the loss of erbB-2 protein observed in western blots is due to an increased degradation rate. EGF induced turnover of erbB-2 is greatly decreased ($t_{1/2}$=28 hours) in the overexpressing cell line, ce2, consistent with the western blot analysis of receptor downregulation. RT-PCR analysis of mRNA levels also indicates that EGF does not affect the transcription rate of erbB-2. (data not shown) The time course of erbB-2 degradation is similar to that previously characterized for the EGF-R in several different cell types (57). Trafficking of the EGF-R to the lysosomes for degradation is an integral part of its downregulation program (57). This data lends further credence to a model in which lysosomal degradation is an important mechanism of erbB-2 negative regulation.

Because our system employs transactivation of erbB-2 by EGF-R, it seemed possible that there were insufficient EGF-R in the ce2 line to fully transmodulate erbB-2. To investigate this possibility, both parental and overexpressing cell lines were treated with EGF for 5 minutes. ErbB-2 was then immunoprecipitated followed by western blot analysis for phosphotyrosine as an indicator of receptor activation status. The degree of erbB-2 phosphorylation was greater in the ce2 cell line than the parental (data not shown). This result indicates that overexpression of erbB-2 did not impair the efficiency of activation via the EGF-R. Interestingly, the basal level of activated erbB-2 was increased in the overexpressing cell line relative to the parental (Fig. 12B).

<u>The effect of erbB-2 overexpression on downregulation of the EGF-R</u> Given the dramatic affect of erbB-2 amplification on EGF induced erbB-2 degradation, the downregulation of the EGF-R was investigated. Initial studies measured the EGF-R level by western blot following 24 hour treatment with EGF. Surprisingly, we observed an increased amount of EGF-R remaining after 24 hours in the erbB-2 overexpressing cells. (Fig. 10A) To further investigate this, we measured EGF-R downregulation by western blot over a shorter time course of exposure to EGF (Fig. 10B). Analysis reveals that EGF induced loss of EGF receptor protein is reduced in cells containing amplified erbB-2. After 2 hours, EGF-R is downregulated 65% in MTSV, while it is downregulated only 30% in ce2. Recall that although erbB-2 receptor levels are increased 6 fold in ce2 relative to MTSV, the EGF-R expression remains constant. Thus, erbB-2 amplification interferes not only with its own degradation but with the downregulation of EGF-R, as well. Although EGF-R downregulation is significantly impaired in ce2 cells, the degree of inhibition is not as great as observed for erbB-2. It is possible that lysosomal

targeting is intrinsically more efficient for the EGF-R than erbB-2, which may explain some of the discrepancies between reports on erbB-2 downregulation.

As previously discussed, downregulation of the EGF-R by intracellular trafficking is regulated at two discrete steps: endocytosis and lysosomal targeting (57). We sought to determine the mechanistic level at which erbB-2 amplification affected EGF-R downregulation. The endocytic rate constant for the EGF-R was measured in the two cell types to determine if erbB-2 overexpression affected this (56). Cells were incubated with radiolabeled EGF over a five minute time course, followed by measurement of both the cell surface associated radioactivity and internalized radioactivity. The data in Fig. 11 is plotted to calculate the internalization rate constant, k_e. We found that overexpression of erbB-2 did not significantly alter the internalization rate constant for the EGF-R (0.21 compared to 0.19 min^{-1}). Another possibility, is that the transcription rate for the EGF-R is increased when erbB-2 is overexpressed. RT-PCR analysis show that the level of EGF-R transcript is unchanged by either overexpression of erbB-2 or treatment with EGF (data not shown). Thus, the effect of erbB-2 amplification on EGF-R degradation appears to occur after endocytosis, and during trafficking from the endosome to the lysosome.

ErbB-2 overexpression increases basal activation levels of erbB-2 and EGF-R

Initial experiments analyzing the activation status of erbB-2 in overexpressing cell lines revealed a significant level of phosphorylated erbB-2 in the absence of EGF. Significantly, the oncogenic version of erbB-2 (neu) contains a point mutation in the transmembrane domain which results in constitutively active receptors, attributed to the constitutive homodimerization seen in this receptor mutant. Because we observed that the effects of overexpressing erbB-2 extended to the EGF-R, we wondered if the basal activation state of the EGF-R would be increased in ce2 vs. parental MTSV cells. Cells were treated with EGF for 5 minutes, then the EGF-R was immunoprecipitated followed by western blot for phosphotyrosine content (Fig. 12B). Interestingly, the basal level of EGF-R activation was increased, indicating the high level of erbB-2 expression affects the activation status of the EGF-R pool. This is intriguing because the level of EGF-R is unchanged, yet the amount of tyrosine phosphorylated receptor is greatly increased. The blot in Fig. 12B also shows that EGF treatment of ce2 cells results in an increase in the level of activated EGF-R, as compared to the MTSV cells. Although the increase in basal activation of the EGF-R was consistent over 5 separate experiments, the increase following EGF treatment was not consistently obtained. The mechanism responsible for this ligand independent activation is currently under investigation in our laboratory.

This study extends our previous results demonstrating downregulation of erbB-2 following transactivation with EGF (68). We measured the loss of receptor mass by western blot following a time course of activation with EGF. erbB-2 is downregulated 75% within 6 hours for the two cell types analyzed here. To confirm that downregulation is indeed due to increased receptor degradation we measured the half-life of erbB-2 in the presence and absence of EGF. While the half-life is 6 hours in the parental MTSV cell lines under control conditions, the half-life is reduced to 0.7 hours when EGF is present. Together these data demonstrate that erbB-2 trafficking is altered following transactivation with EGF, resulting in downregulation of the receptor. We propose that intracellular trafficking is an effective means of downregulating erbB-2 analogous to that previously described for the EGF-R.

Conclusions

We are making excellent progress on our project. It has been appreciated that growth factors provide important information to the cell regarding its environment. They can also stimulate mitogenesis. Understanding how growth factors and their receptors are regulated in normal cells is essential to understand aberrant regulation in cancer. Our work continues to show that the normal spatial distribution and trafficking of both growth factors and their receptors are necessary for normal cell behavior and physiology. Our studies are unique in that they focus on the expression of biologically active receptor and ligand proteins in nontransformed cells that depend on these proteins for their function. By continuing to look at this physiologically relevant context, we will gain a deeper insight into the significance of different aspects of receptor regulation.

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Figure Legends

Figure 1. Panel A. Overexpression of EGFR in LLCPK1 Cells results in high receptor expression at both the apical and basolateral surfaces. Cells were incubated to equilibrium at 0°C with saturating concentrations of ^{125}I-EGF (12 nM) applied to either the apical (Ap) or basolateral surfaces (BL). Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled EGF. Insert is scaled data from the wild type (WT) LLCPK1 cells. Panel B. Total phosphotyrosine of K2 cells treated with EGF at either the apical or basolateral surfaces. EGF (15 nM) in complete medium was added to either the apical (Ap) or the basal (BL) chamber . Shown is a western blot of total cellular phosphotyrosine detected using antibody PY20, followed by a rabbit-anti-mouse monoclonal antibody, and detected with ^{125}I-protein A.

Figure 2. Panel A. Mitogenic response of polarized K2 cells to EGF applied to the apical or basolateral surfaces. Following EGF addition at 0 time to the indicated transwell chamber, acid insoluble cpm were measured between 18 and 22 hrs. In some cases, the anti-EGFR antagnonistic monoclonal antibody 528 was added at 0 time to the indicated chamber. Panel B. SHC phosphorylation in response to either apical or basolateral treatment with EGF. Polarized monolayers were treated with 15 nM EGF for 10 min at 37°C, SHC was immunoprecipitated and separated by gel electrophoresis. Phosphotyrosine in the samples was detected by western blot analysis as described in Fig. 1.

Figure 3. Internalization and down regulation of EGFR from apical and basolateral surfaces. Panel A: Polarized monolayers of K2 cells on transwell inserts were incubated with 10 ng/ml of ^{125}I-EGF (150,000 cpm/ng) added to either the apical (—O—) or basolateral (—●—) chambers. At 1 min intervals, the relative amount of internalized and surface associated EGF was measured and converted to internalization plots. Nonspecific EGF binding was determined in parallel using a 1000-fold excess of unlabeled EGF. Panel B: LLCPK1 cells expressing the c'973 EGFR. Polarized monolayers were evaluated for internalization from the apical versus basolateral surfaces as described above for panel A. panel C: down-regulation of surface EGFR from apical versus basolateral compartment. Polarized monolayers of K2 cells were incubated with 100 ng/ml unlabeled EGF added to either the apical (—O—) or basolateral (—●—) chamber at 37°C for the indicated periods of time. Shown in the amount of radiolabeled EGF bound as a percent of that bound to untreated cells.

Figure 4. Differential phosphorylation of FAK and beta-catenin in polarized monolayers. Polarized monolayers of K2 cells were treated with or without EGF added to either the apical or basolateral chambers. FAK antibodies (panel A) or beta-catenin mAb (panel B) were added to the lysates to immunoprecipitate the respective proteins which were separated on SDS gels, transferred to nitrocellulose, then probed with either antiphosphotyrosine mAb (left panels), or beta-catenin or FAK mAb (right panels).

Figure 5. Scatchard analysis of parental 184A1 HMEC and the AXR1 derivative. Confluent monolayers were incubated at 0°C with EGF concentrations ranging from 0.1 to 200

ng/ml to equilibrium (18hr). The specific binding is presented as a Scatchard plot.

Figure 6. AXR1 derivative of HMEC still depend on EGFR activation for maximal growth. Approximately 30,000 of either parental 184A1 cells (top panel) or the AXR1 derivative (bottom panels) were plated into 35 mm dishes and incubated with either EGF or 10μg/ml 225 mAb. The cell numbers on the indicated days was determined for each group.

Figure 7. erbB-2 downregulation is inhibited by erbB-2 overexpression. Panel A. erbB-2 downregulation was assessed by western blot from cells treated with EGF for 24 hours. B. Downregulation of erbB-2 was measured over a shorter time course (6 hr). Western blot bands were quantitated by phosphorimager and averages from three experiments are plotted as a percent of control. The MTSV cell line downregulate EGF-R 70% within 6 hours, while the erbB-2 overexpressing cell line (ce2) downregulates EGF-R only 35% by 6 hours. C. Kinetics of downregulation. The amount of erbB-2 at the indicated times after EGF treatment was determined by western blot analysis.

Figure 8. erbB-2 degradation pathway is saturated in ce2 cells. Data from figure 1A. was recalculated to give the amount of erbB-2 lost in 24 hours. The signal from cells treated with EGF was subtracted from the signal from control cells. The amount of erbB-2 degraded in 24 hours is plotted in phosphorimager units. ce2 cells degrade approximately the same receptor mass as MTSV cells. This indicates that the additional activated receptors in ce2 cells are not degraded, resulting in less efficient receptor loss.

Figure 9. Inhibition of erbB-2 downregulation is at the level of degradation. The indicated cells were pulse-labeled with [^{35}S]methionine and treated either without or with EGF for the indicated time. The erbB-2 was immunoprecipitated, separated by gel electrophoresis and quantified using a phosphoimager.

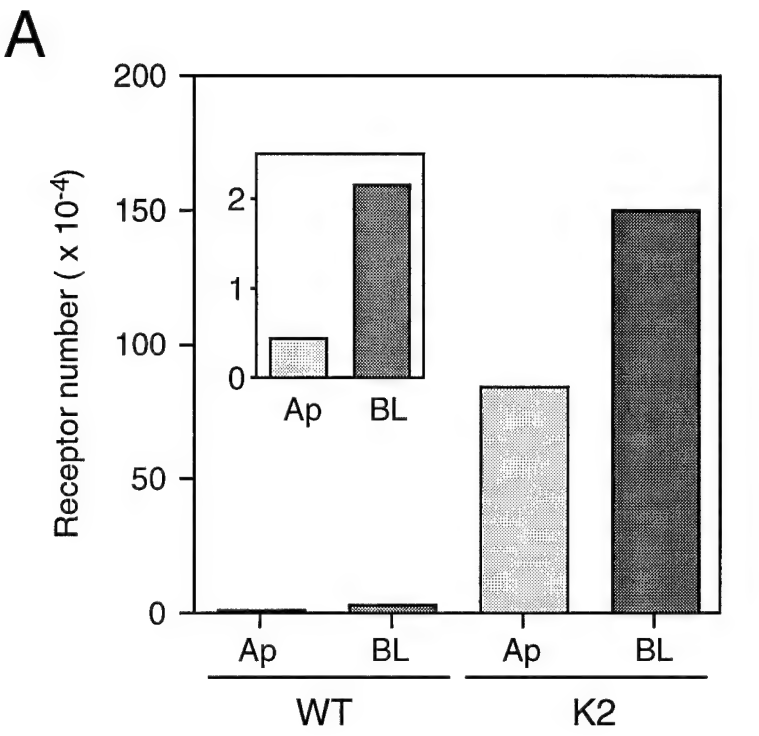
Figure 10. EGF-R downregulation is inhibited by erbB-2 overexpression A. EGFR downregulation was assessed by western blot for EGFR from cells treated with EGF for 24 hours. B. Kinetics of downregulation of the EGFR over a 24 hr time course. Western blot bands were quantitated by phosphorimager and averages from three experiments are plotted as a percent of control. The MTSV cell line downregulate EGFR 70% within 6 hours, while the erbB-2 overexpressing cell line (ce2) downregulates EGFR only 35% by 6 hours.

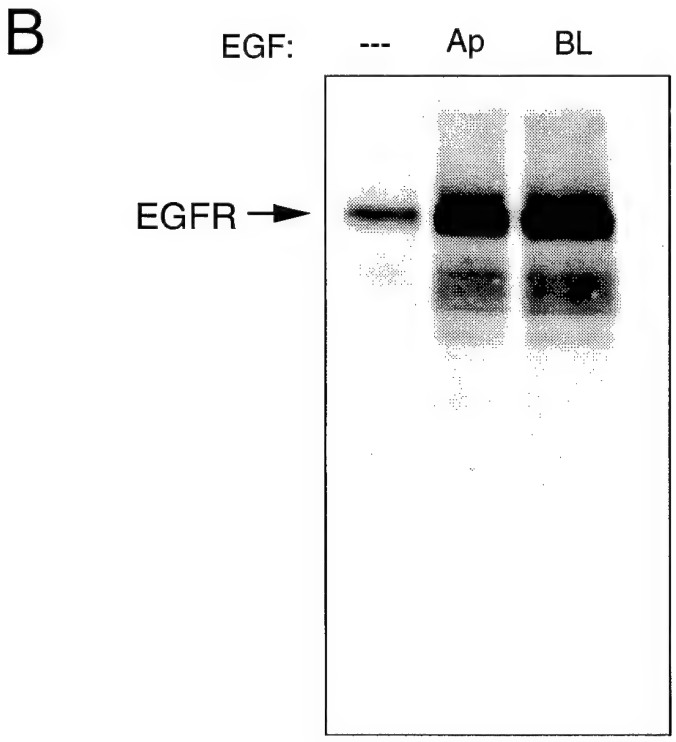
Figure 11. EGF-R internalization is unaffected by erbB-2 overexpression. Radiolabeled EGF is used to follow internalization of the EGFR over a five minute time course. Internalized CPM vs. surface bound CPM is plotted for MTSV and the erbB-2 overexpressing cell line, ce2.

Figure 12. Activation of erbB-2 and EGFR in MTSV vs. ce2 Panel A: Cells were treated with EGF for 0 or 5'. erbB-2 was immunoprecipitated followed by western blot for phosphotyrosine (PY) Cells overexpressing erbB-2 (ce2) yielded more activated erbB-2 as assessed by phosphotyrosine content. This indicates that activation of erbB-2 is not inhibited in cells overexpressing erbB-2. Additionally, there is a substantial level of

activated erbB-2 in ce2 in the absence of EGF treatment. Panel B :Following erbB-2 immunoprecipitation, EGFR was immunoprecipitated from the extracts in part A. The amount of activated EGFR was determined by western blot for phosphotyrosine content. Cells overexpressing erbB-2 (ce2) contain activated EGFR in the absence of EGF treatment.

Figure 1

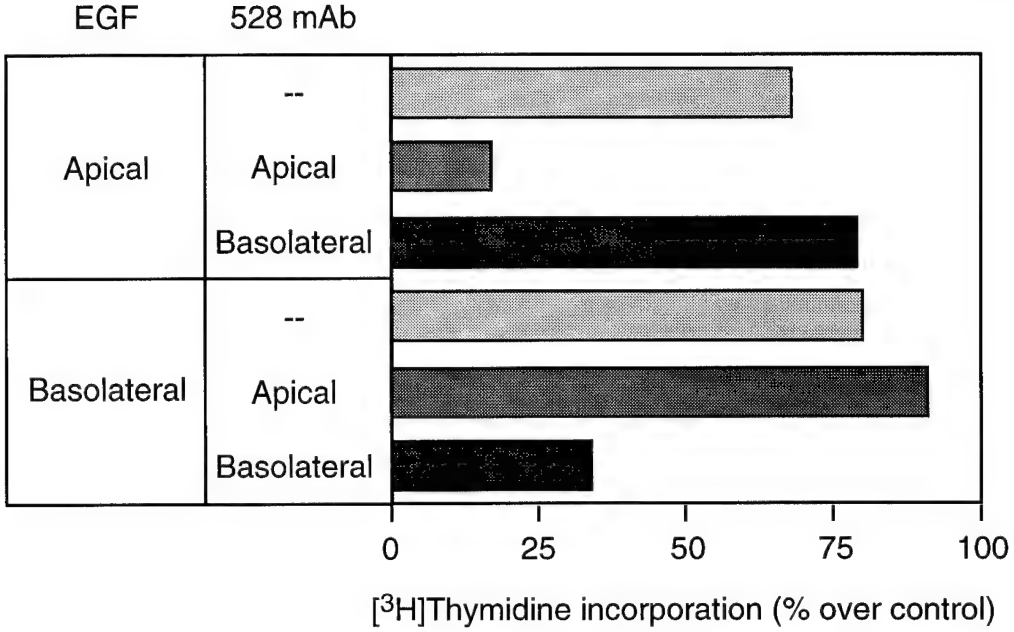

A
Receptor number (x 10-4)
200
150
100
50
0
2
1
0
Ap
BL
Ap
BL
Ap
BL
WT
K2


B
EGF:

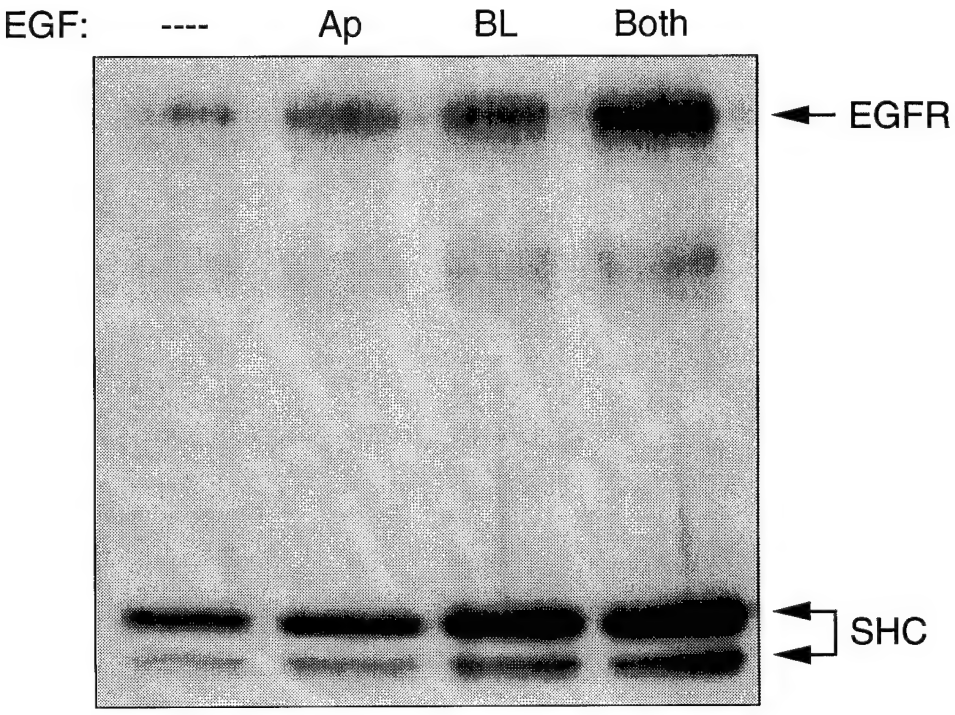
Ap
BL
EGFR

Figure 2

A


EGF
528 mAb
Apical
--
Apical
Basolateral
Basolateral
--
Apical
Basolateral
0
25
50
75
100
[3H]Thymidine incorporation (% over control)

B


EGF:

Ap
BL
Both
EGFR
SHC

Figure 3A

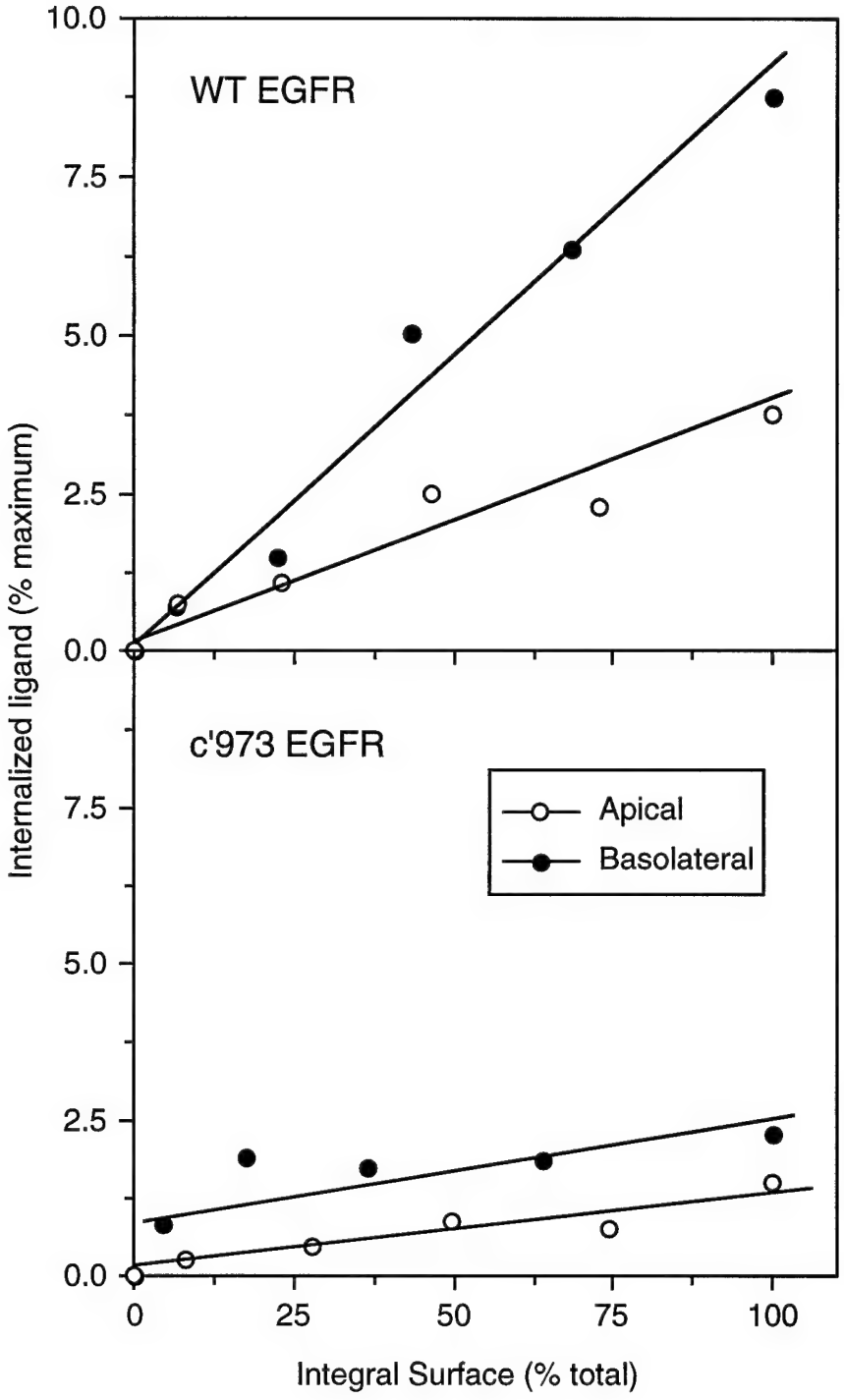

WT EGFR
c'973 EGFR
Apical
Basolateral
Internalized ligand (% maximum)
Integral Surface (% total)
10.0
7.5
5.0
2.5
0.0
0
25
50
75
100

Figure 3B

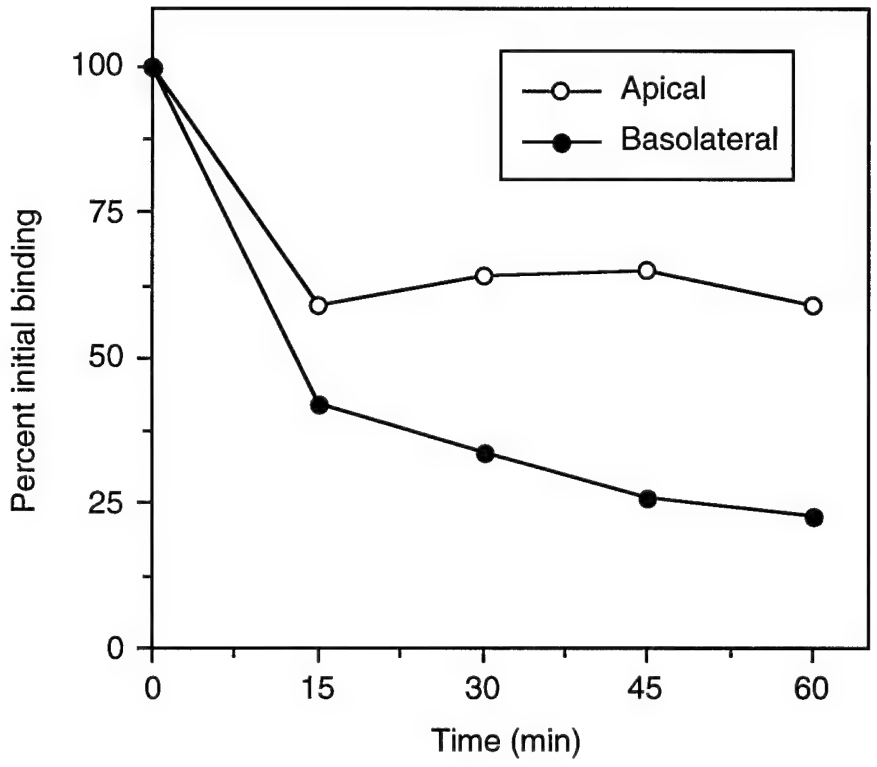

Apical
Basolateral
Percent initial binding
100
75
50
25
0
0
15
30
45
60
Time (min)

Figure 4

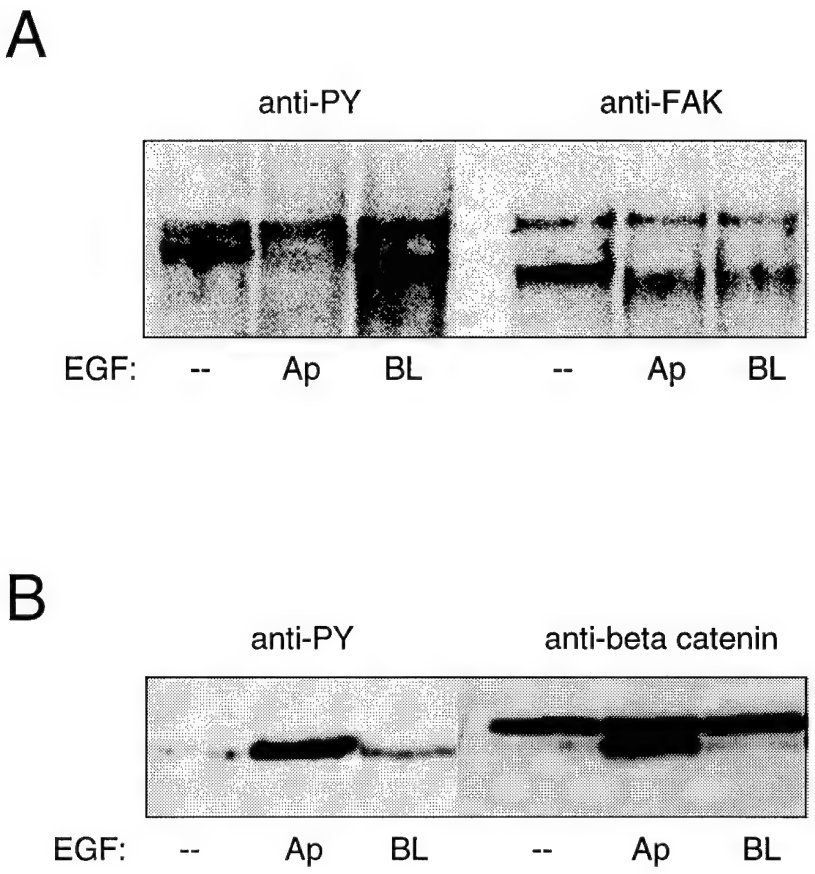

A
anti-PY
anti-FAK
EGF: -- Ap BL -- Ap BL
B
anti-PY
anti-beta catenin
EGF: -- Ap BL -- Ap BL

Figure 5

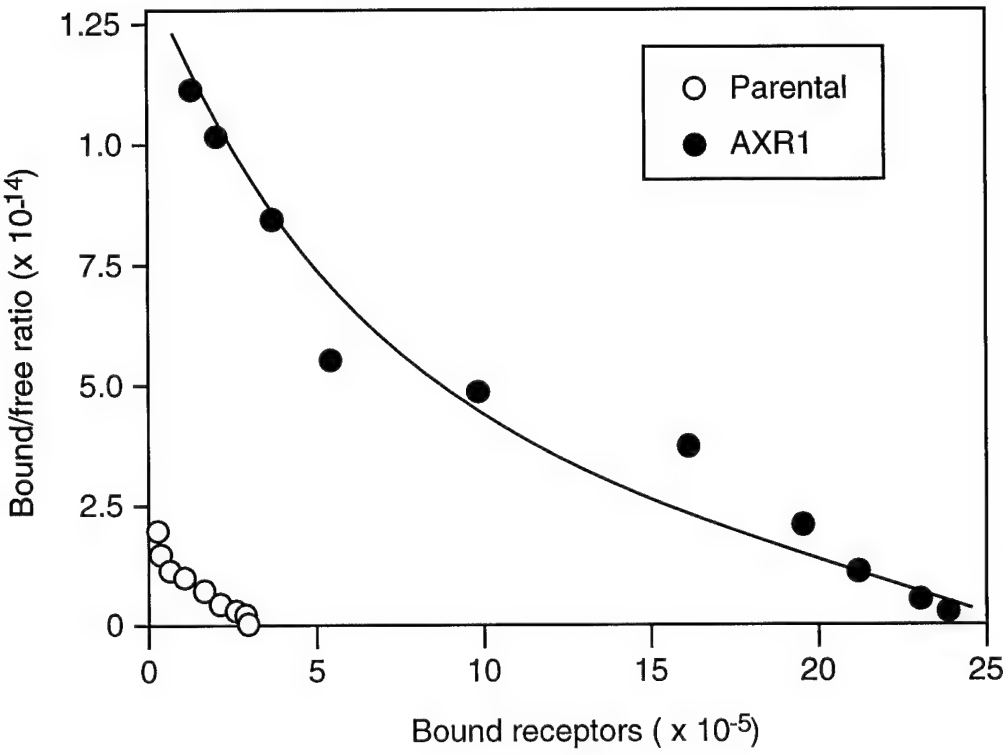

1.25
1.0
7.5
5.0
2.5
0
Bound/free ratio (x 10-14)
0
5
10
15
20
25
Bound receptors (x 10-5)
Parental
AXR1

Figure 6

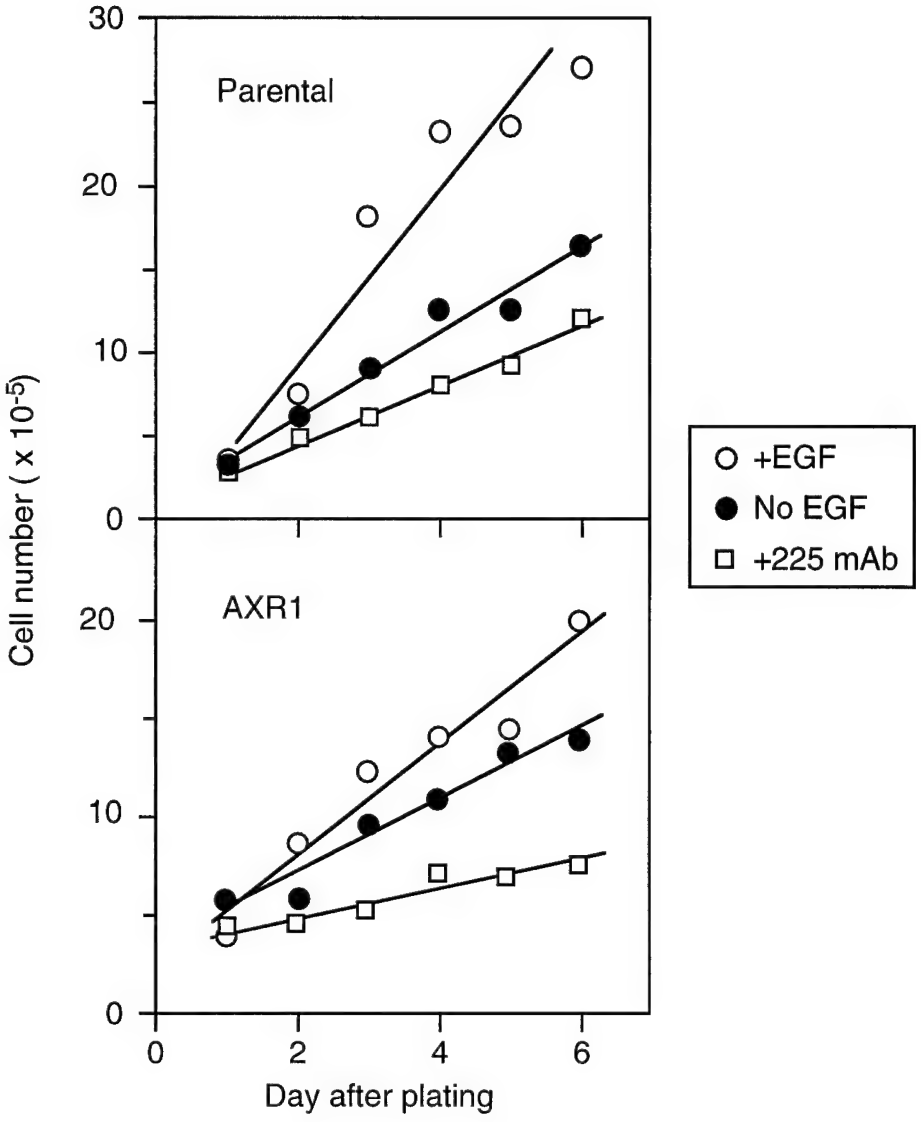

Parental
AXR1
Cell number (x 10-5)
Day after plating
+EGF
No EGF
+225 mAb
0
10
20
30
0
2
4
6

Figure 6

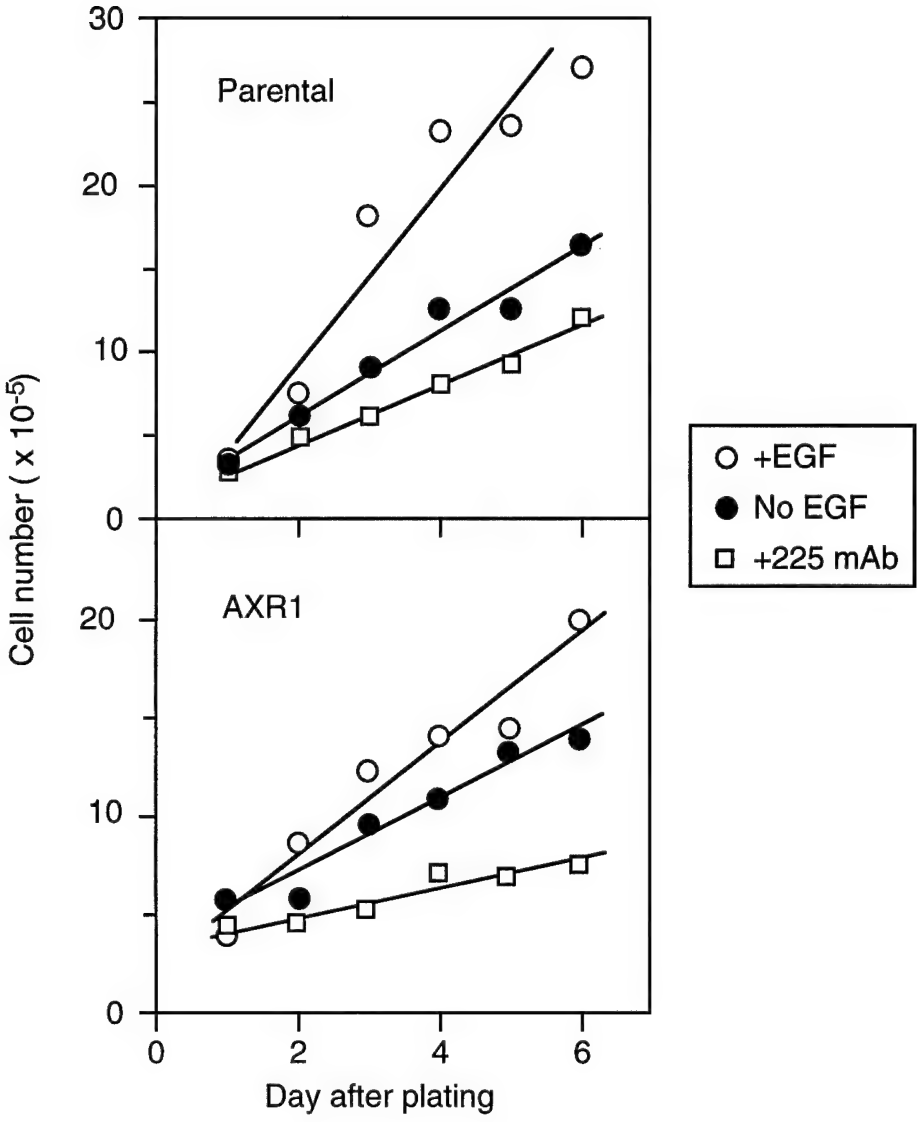
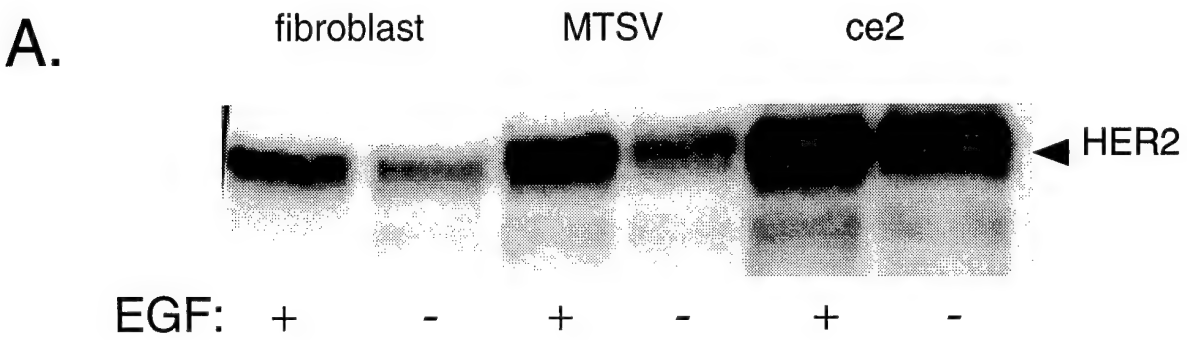
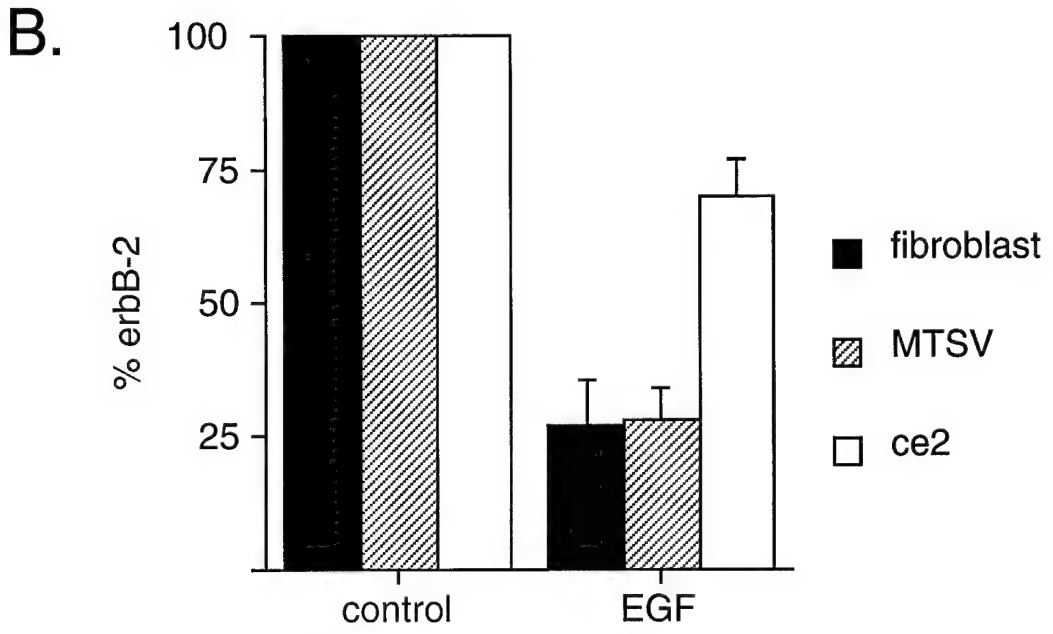

Parental
AXR1
Cell number (x 10-5)
Day after plating
+EGF
No EGF
+225 mAb
30
20
10
0
0
2
4
6

Figure 7


A.
fibroblast
MTSV
ce2
HER2
EGF: + - + - + -


B.
100
75
50
25
% erbB-2
control
EGF
fibroblast
MTSV
ce2

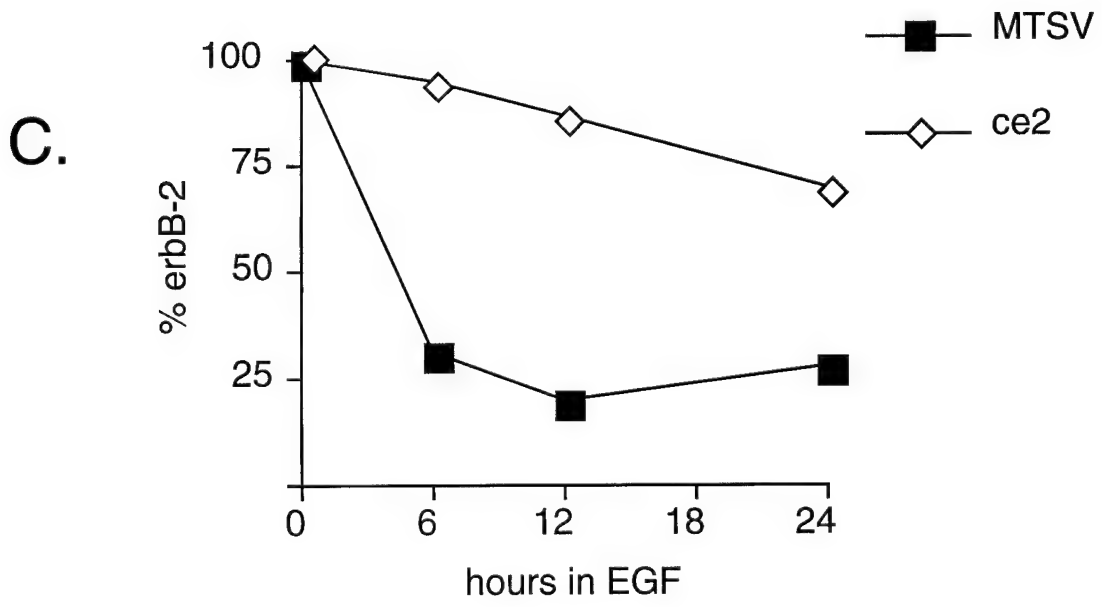

C.
MTSV
ce2
100
75
50
25
% erbB-2
0
6
12
18
24
hours in EGF

Figure 8

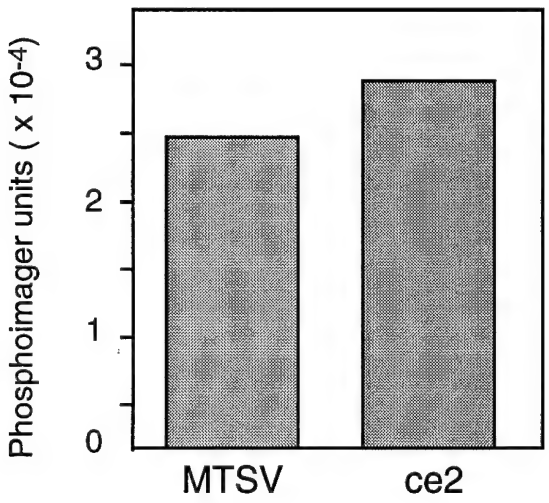


Figure 9

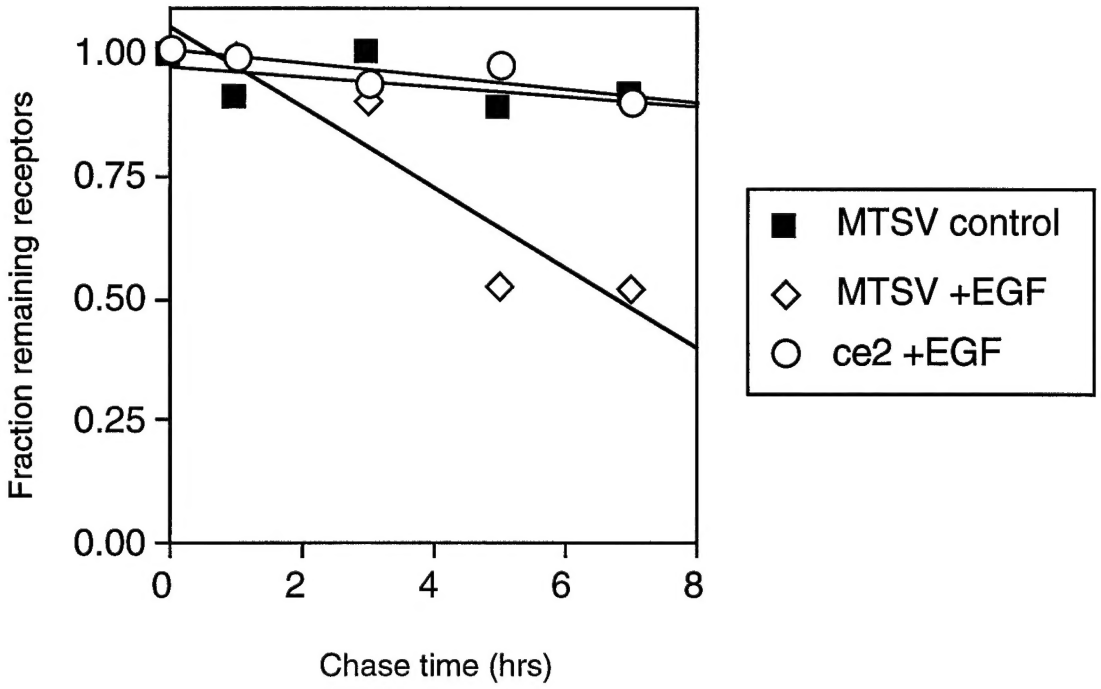
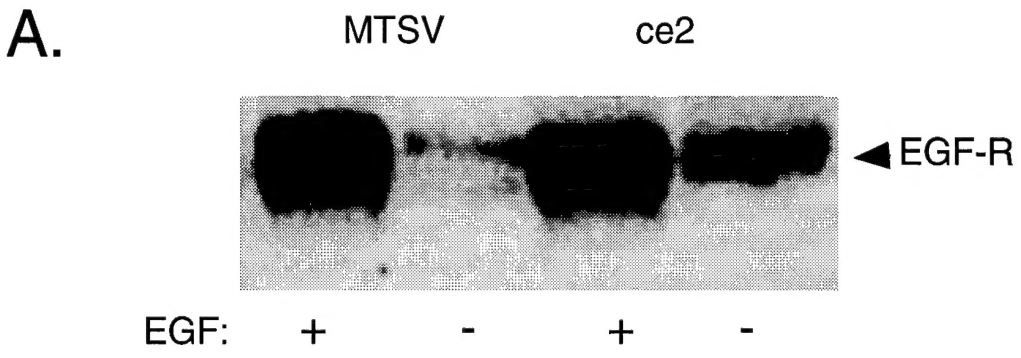

Fraction remaining receptors
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
Chase time (hrs)
MTSV control
MTSV +EGF
ce2 +EGF

Figure 10


A.
MTSV
ce2
EGF-R
EGF:
+
-
+
-

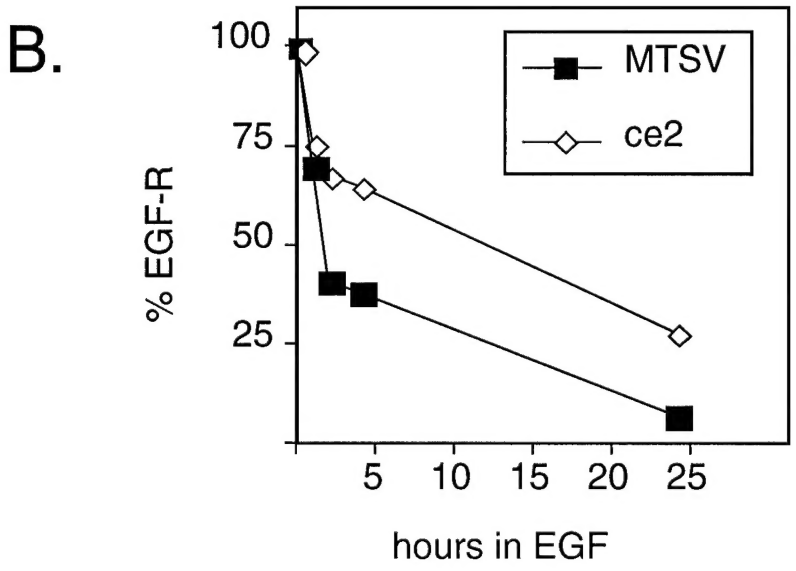

B.
100
75
50
25
% EGF-R
MTSV
ce2
5
10
15
20
25
hours in EGF

Figure 11

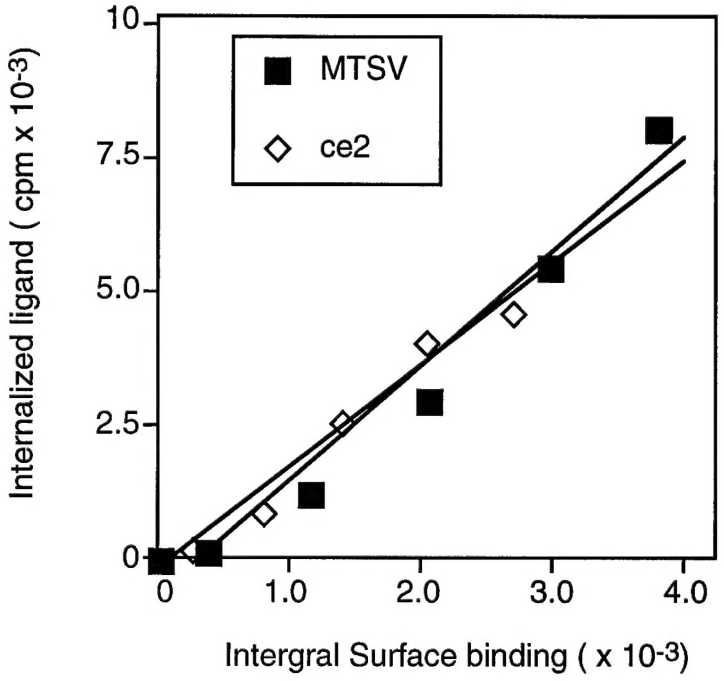

MTSV
ce2
Internalized ligand (cpm x 10-3)
Intergral Surface binding (x 10-3)
10
7.5
5.0
2.5
0
0
1.0
2.0
3.0
4.0

Figure 12